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Control of Stereoselectivity in an Enzymatic Reaction by Backdoor Access**

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The ability of biopolymers to discriminate between optical isomers is vital for living systems, and the selective formation of only one product stereoisomer from achiral substrates is one of the most sophisticated tasks for enzymes. Using a Diels–Alder ribozyme as an example, we demonstrate here that by a simple strategy a biocatalyst can be used to selectively synthesize both product stereoisomers in one catalytic pocket, namely, by controlling access to the active site from opposite directions through different “doors”. While substrates tethered to the catalyst were used to observe this phenomenon, we propose

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that nature may use conformational gating to control the stereoselectivity of enzymatic reactions.

Since the importance of stereoselective recognition to biology was realized, the structural and mechanistic basis underlying these phenomena has become the subject of intensive research.^[1–4] Enzymatic reactions provide the basis of cellular biochemistry and typically display high stereoselectivity. Enzyme engineering and in vitro evolution not only aid in understanding the structural basis of stereoselectivity but they also provide tools to manipulate this property.^[5–7] The standard view of an enzyme's active site is that of a pocket with an opening on one side and an array of functional groups that precisely match molecular features of the reaction's transition state. Recent results, however, indicate that many enzymes contain more than one possible entrance to the active site, but the function of these “alternative entrances” or “backdoors” remains unclear.^[8–10] They have been implicated in controlling substrate entrance, product egress, removal of bound water, cofactor binding, and proton shuttling.^[11,12] The stereochemical consequences of having multiple-access pathways to one stereoselective catalytic center, however, have not been investigated so far.

Our laboratory previously discovered an RNA enzyme (ribozyme) that catalyzes the formation of carbon–carbon bonds by a Diels–Alder reaction between an anthracene diene and a maleimide dienophile (Figure 1a).^[13] This reaction type is of great relevance in organic chemistry,^[14] and candidates for catalyzing the Diels–Alder reaction in nature are currently under intensive investigation.^[15–17] This ribozyme was the first RNA enzyme to catalyze a bond-forming reaction enantioselectively,^[18] and substrate-specificity studies suggested a simple and convincing structural model for the stereoselectivity (Figure 1b).^[19] The size of the diene's substituent was identified as the major determinant of stereoselectivity (see the table in Figure 1), and the diene was thought to enter the catalytic pocket with the sterically less demanding edge first, then react with the maleimide bound in one fixed orientation (Figure 1b, left model), while the opposite orientation of the anthracene (right model) was found to be disfavored.

Rather than a typical enzyme pocket, the recent X-ray crystal structure of the ribozyme/Diels–Alder product complex surprisingly featured a catalytic center accessible from both front and back sides, with two openings of different sizes. In fact, the reaction product was bound inside the pocket with the sterically more demanding side first, contrary to what had been expected (Figure 1c).^[20] The only difference between these and the previous experiments was that the substrate specificity was investigated using free substrates (true catalysis, Figure 2a, I), while for co-crystallization, the Diels–Alder product was covalently linked to the ribozyme by an 18-atom flexible tether (reaction with a tethered substrate, Figure 2a, II) which was attached to the RNA close to the backdoor. This raised the question whether restriction of the substrate's translational and rotational mobility by tethering could force it to enter the catalytic pocket through the narrower, disfavored backdoor and thus influence the stereochemistry of the reaction. Our investigation of this phenomenon provides direct chemical evidence that in the true catalytic reaction and the tethered version, the substrates use different approaches to the catalytic pocket, bind in different orientations, and are converted to yield the opposite product enantiomers.

To study the influence of tethering on stereoselectivity, a chromatographic assay was established. A Diels–Alder reaction of *N*-pentylmaleimide with anthracene attached to the ribozyme's 5'-end by a hexa(ethylene glycol) tether was carried out, and the reaction products were digested by snake venom phosphodiesteraseI to liberate the hexa(ethylene glycol)-tethered reaction product(s). In parallel, the reaction mixture for the true catalysis reaction derived from chemically identical hexa(ethylene glycol)anthracene, *N*-pentylmaleimide, and ribozyme, as well as an uncatalyzed background reaction (hexa(ethylene glycol)anthracene + *N*-pentylmaleimide) were investigated under otherwise identical conditions. The resulting product mixtures were then analyzed by HPLC on a chiral stationary phase (Figure 2a,b).^[18] While the background reaction gave the racemic mixture (Figure 2b, black curve), both catalyzed reactions produced one product in large excess (over 90% *ee*). Whereas the true catalytic reaction produced predominantly the *R,R* enantiomer (blue curve), the product of the tethered version was found to be the *S,S* enantiomer (red curve), thereby supporting our initial assumption.

According to the crystal structure, the attachment site of the tether is located directly behind the backdoor, and measured through the door, the distance between 5'-phosphate and anthracene's 9'-O is only 7 Å. Diene binding through the frontdoor by folding the tether around the ribozyme's backbone would require a tether length of ≈ 35 Å to reach the pocket. As the length of a hexa(ethylene glycol) tether in a fully stretched conformation is only 21 Å, these data imply that in the tethered version, the tether must be threaded through the backdoor, leading to the orientation observed in the crystal structure. According to this hypothesis, the preference for the frontdoor over the backdoor should depend on the tether length, and Figure 2c shows the systematic investigation of both reaction formats for tether lengths between 0 and 12 ethylene glycol units.

EG₀ (no tether; 9-hydroxymethylanthracene directly esterified with the RNA 5'-phosphate) is not accepted as a substrate, as the diene is apparently unable to reach the catalytic center. Between EG₂ and EG₈, the tethered version always gives *ee* values between 90 and 95% in favor of the *S,S* enantiomer, consistent with the assumption of threading through the backdoor (Figure 2C, blue curve). At EG₁₀ (extended tether length: 35 Å), the *ee* value drops to 6% (*S,S*), while at EG₁₂ (extended tether length: 42 Å) the stereoselectivity is inverted (51%, in favor of the *R,R* enantiomer). Apparently, the tether is now long enough for the anthracene to reach the frontdoor which is preferred owing to the larger size of its opening.^[21] For the true catalytic reaction (Figure 2c, red curve), however, the *R,R* product is always favored, and the selectivity increases with the size of the substituent.^[22]

These data imply the following mechanism of stereoselection (Figure 2d):

- In the true catalytic reaction, the free diene enters the catalytic pocket with the sterically less demanding side first through the wide frontdoor (longest dimension: ≈ 10 Å), as proposed previously,^[18,19] resulting in the formation of the *R,R* enantiomer.
- If the diene is attached to the RNA by a short tether, this tether threads through the smaller door in the back of the pocket (longest dimension: ≈ 6 Å), giving rise to the opposite product enantiomer. The mechanism by which the tethered anthracene

(shortest dimension: ≈ 7 Å) gets through the narrow hole is currently unknown; most likely dynamic opening and closing of the ribozyme's tertiary base pairs enlarges the door and facilitates threading.^[23]

- If the tether is long enough (35 Å), it folds around the RNA backbone and the diene binds like in the true catalytic reaction. It has been shown previously for tethered ligand–receptor pairs that even nearly stretched tether conformations lead to productive binding events if sufficient sampling time is permitted.^[24]

The most important aspect of this investigation is the finding that one evolved enzyme active site can be used for the selective synthesis of both product enantiomers, depending on which entrance to the catalytic center the substrate is permitted to use.^[25] Although the situation created for observing this phenomenon (covalent tethering of catalyst and substrate) is an artificial one, this discovered mechanism of stereoselection raises the question whether nature might use similar strategies for “dual use” of active sites, providing controlled synthesis of both product enantiomers using only one enzyme. Alternative access pathways and backdoors have been suggested in key enzymes of metabolism (cytochrome P450s),^[9] signal transduction (acetylcholine esterase),^[26] and muscle action (myosin).^[27] The regulation of different access pathways to the active center might be achieved by conformational changes or by binding of effectors, which are both standard gating mechanisms, or in the case of multienzyme complexes, alternative substrate channeling strategies might be used, thereby allowing for adaptation to changing metabolic needs. Like enzymes that catalyze different reactions (“catalytic promiscuity”)^[28] or carry out completely different functions (“moonlighting”),^[29,30] the regulated synthesis of different stereoisomers may provide an evolutionary more simple and economic strategy than the de novo evolution of a new catalyst.^[31–33]

Supplementary Material

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Figure 1.

Ribozyme-catalyzed Diels–Alder reactions. a) Bimolecular reaction between oligo(ethylene glycol)anthracene derivatives and *N*-pentylmaleimide catalyzed by a ribozyme. Enantioselectivity is controlled by the ethylene glycol substituent of the anthracene substrate. *R,R* and *S,S* denote the stereochemical configuration at the carbon atoms with asterisks. b) Model for the stereoselection in the RNA-catalyzed reaction with free substrates; O atoms in red, N atoms in blue. Preferred orientation (left), disfavored orientation (right). The chart on the right shows the *ee* values for the enantioselective formation of *R,R* product with different substituents on the anthracene unit. c) Surface representation of the Diels–Alder ribozyme crystal structure; P atom in green, O atoms in red; view from the front side (left), view from the back side (middle), enlarged picture of the backdoor with assigned distance between the G1-phosphate group and the Diels–Alder product inside the pocket.

Figure 2.

Investigation of the ribozyme's stereoselectivity in a true catalytic reaction (I) and in a reaction with a tethered substrate (II). a) Reaction scheme. b) HPLC chromatograms of the reaction mixture: background reaction without ribozyme (black), reaction with anthracene covalently attached to the Diels–Alder ribozyme by a hexa(ethylene glycol) tether (blue), and catalytic reaction of the free substrates in presence of the Diels–Alder ribozyme (red). c) Dependence of the ribozyme's stereoselectivity on the number of ethylene glycol (EG) units in the tethered version (blue) and in the catalytic reaction (red). d) Models for the mechanism of stereoselection. For experimental details, see the Supporting Information.